Applied and Environmental Microbiology, June 2008, p. 3804–3811 Vol. 74, No. 12
0099-2240/08/$08.00+0 doi:10.1128/AEM.02537-07

Effect of Extraction and Assay Media on Analysis of Airborne Endotoxin▽

Suzanne Spaan,[1,2] Gert Doekes,[1] Dick Heederik,[1] Peter S. Thorne,[3] and Inge M. Wouters[1]*

Institute for Risk Assessment Sciences (IRAS), Environmental Epidemiology Division (EEPI), Utrecht University, Utrecht, The Netherlands[1]; Quality and Safety Business Unit, Food and Chemical Risk Analysis Division, TNO Quality of Life, Zeist, The Netherlands[2]; and Environmental Health Sciences Research Center, University of Iowa, Iowa City, Iowa[3]

Received 10 November 2007/Accepted 18 April 2008

The measurement of airborne endotoxins is thus far not standardized. Earlier studies reported higher endotoxin yields when Tween 20 was added to the media used for filter extraction and in the *Limulus* amebocyte lysate (LAL) assay. This study compared four common media and assessed the effects of Tween during extraction and analysis separately. Parallel airborne dust samples from five work environments ($n = 250$) were used to compare the four media (pyrogen-free water [PFW], PFW-Tween 20, PFW-Tris, and PFW-triethylamine-phosphate [TAP]) and an extraction time of 10 or 60 min. A subset of the extracts in PFW or PFW-Tween ($n = 40$) were analyzed in parallel LAL assays with PFW or PFW-Tween as the assay medium. The results produced by a shorter extraction time or the presence of Tris were similar to the results for the reference procedure (PFW and 60 min of shaking). The use of PFW-TAP showed overall lower yields and a deviant calibration curve. The presence of Tween in the extraction medium resulted in significantly ($P < 0.05$) higher endotoxin yields from all dust types, independent of the effect of Tween in the assay. Tween in the LAL assay, however, also strongly inhibited the reactivity of the lipopolysaccharide (LPS) standard, thus shifting the calibration curve to higher values. The inhibition of LPS in test samples was less pronounced and varied between dust sources, resulting in enhanced calculated concentrations. This assay effect could be circumvented by diluting extracts at least 50-fold before the LAL assay. In conclusion, of the media tested, only Tween enhances the efficiency of endotoxin extraction from airborne dust samples in a consistent manner. We recommend extraction in PFW-Tween combined with dilution and LAL analysis in PFW.

Endotoxins are lipopolysaccharide components (LPS) of the cell wall of gram-negative bacteria with a high proinflammatory potency. Exposure to airborne endotoxins has been associated with the development of nonallergic asthma, bronchitis, organic dust toxic syndrome, and accelerated lung function decline in a variety of agricultural and industrial environments (22). However, endotoxin exposure in early childhood is associated with a lower prevalence of atopy and allergic disease, especially in farm children (11, 29), and studies of adult working populations suggest that it may also protect against atopic sensitization at a later age (11, 18).

In order to compare results from studies investigating endotoxin exposure, related health effects, and compliance with possible exposure limits, the exposure assessments should be comparable. Although the *Limulus* amebocyte lysate (LAL) assay is part of most common procedures for endotoxin exposure assessment, the procedure is not completely standardized. Guidelines for exposure assessment, like those published by the European Committee for Standardization (CEN) (3, 4), are in fact only partially based on systematically collected empirical data, which leaves room for variation in interpretation of the procedure.

The effects of variations in the extraction protocol and/or extraction medium (7, 9, 12–14, 15, 26, 28) or modifications of the LAL assay conditions (12, 13, 16, 28, 30) on the measured endotoxin concentration have been investigated. However, most of these studies investigated only some options in a limited number of samples from a few different types of dust, whereas a previous study showed dust type to be of importance to the outcome (21). We recently studied the influence of and interactions between transport conditions, storage of samples, extraction medium, storage of extracts, filter type, and assay medium on the measured endotoxin concentration in parallel samples from two work environments (23). The extraction medium appeared to be the most important determinant, with higher measured endotoxin concentrations when extraction was done in the presence of 0.05% Tween 20 than in pyrogen-free water (PFW). Although a more-efficient extraction of endotoxin from filters was the most likely explanation, the use of Tween in the medium of the LAL assay also appeared to result in higher endotoxin concentrations. This seemed to be an additive effect, but was only studied in a subset of the samples.

Besides PFW with or without the addition of 0.05% Tween 20, other media have also been used as extraction and assay media (7, 10, 12, 25). Therefore, the current study investigated the effects of several extraction media that are used regularly, as well as the effect of the duration of shaking on the measured endotoxin concentrations in parallel samples from five representative work environments. In addition, experiments were performed to further elucidate the effect of Tween during extraction and/or analysis separately.

MATERIALS AND METHODS

Collection of inhalable dust samples. A parallel sampler, developed within the European MOCALEX project (2, 8), was used to collect air samples in five work environments (grass seed production, pig farm, household-waste composting, potato processing, and sewage treatment) representing different sources of endotoxin exposure. As previously described, the parallel sampler enabled the

* Corresponding author. Mailing address: Institute for Risk Assessment Sciences, Environmental Epidemiology Division, Utrecht University, P.O. Box 80178, 3508 TD Utrecht, The Netherlands. Phone: 31 30 253 9449. Fax: 31 30 253 9499. E-mail: I.Wouters@uu.nl.

▽ Published ahead of print on 25 April 2008.

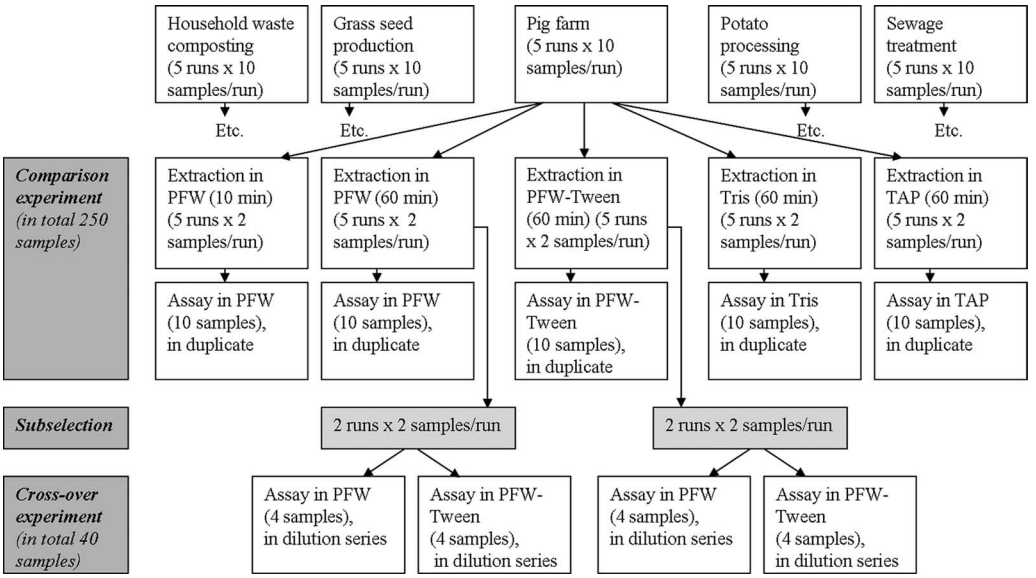

FIG. 1. Schematic overview of the design of the experiments with filter samples.

simultaneous collection of 10 close-to-identical airborne dust samples per run using PAS-6 sampling heads for inhalable dust (24) equipped with 25-mm glass fiber filters (Whatman GF/A, United Kingdom) (23). The loaded filters were stored for 56 to 90 days at −20°C prior to extraction.

A total of 250 samples were collected by performing five runs of 10 parallel samples per work environment. Air samples were collected during 15 days in 2003, with 3 or 4 sampling days per location. The sampling time varied from 1 to 8 h to obtain a sufficient range of dust and endotoxin loads on the filters. On every sampling day, a control filter (field blank) was included which was handled like the test filters except for the actual exposure in the sampler.

Comparison of different media used in both extraction and the LAL assay and of extraction time. In the first experiment, as schematically shown in Fig. 1, the effects of four commonly reported extraction media on the measured endotoxin concentration were evaluated: (i) PFW (Braun, Germany); (ii) PFW-Tween (PFW with 0.05% [vol/vol] Tween 20 [polysorbate 20; Merck, Germany]); (iii) triethylamine-phosphate (TAP) (PFW with 0.05 M K_2HPO_4 [Merck, Germany] and 0.01% triethylamine [Fisher, United Kingdom]), pH 7.5; and (iv) Tris (PFW with 1 mM Tris HCl [Gibco, United Kingdom]), pH 7.4. To assess the effect of the shaking period, the extraction in PFW was done for both 10 and 60 min. Of each series of 10 parallel-collected filters, 2 filters were extracted with each of the five extraction methods. The different treatments were randomly assigned to the 10 parallel sampling positions available per run. Each filter was immersed in 5 ml of the extraction medium in a glass tube and rocked vigorously for 1 h at room temperature on a horizontal shaker (160 reciprocations/min and 15 cm deflection), except for the filters assigned to the procedure of shaking for 10 min in PFW. After 15 min of centrifugation at 1,000 × *g*, 1 ml supernatant per sample was collected and vortexed, and four aliquots of 0.1 ml and the remaining 0.6 ml were stored in pyrogen-free glass tubes at −20°C until analysis.

Effect of Tween in the LAL assay. To assess the effect of Tween on the reactivity of the calibration standard in the LAL assay, two experiments were performed. First, two vials of the LPS standard were dissolved in either PFW or PFW-Tween, serially diluted (12 dilution steps) to the usual concentration range (0.05 to 100 endotoxin units [EU]/ml) in either PFW or PFW-Tween, and tested with the LAL reagent dissolved in either PFW or PFW-Tween, all tested in the same microplate. In a second experiment, eight parallel dilution series of the LPS standard were tested in PFW with Tween concentrations varying from zero to 0.15%.

Crossover analysis of the effects of Tween in extraction or assay medium. A crossover experiment was performed with unused replicate aliquots from a selection of the sample extracts from the comparison study (see Fig. 1). For each of the five work environments, two runs were selected, and from each run were selected four samples which were extracted for 60 min in either PFW or PFW-Tween, resulting in 40 sample extracts. Each extract was tested in parallel in the same microplate, either in PFW as the test medium, i.e., with the sample dilution, LAL reagent, and calibration standard series in PFW, or with the samples, standard, and reagent diluted or dissolved in PFW-Tween.

Analysis. The endotoxin concentrations were measured in pyrogen-free microplates (Costar, Corning, NY) with the kinetic, chromogenic LAL method (lysate lot no. 3L433E and standard lot no. 3L2950 [reference standard endotoxin/control standard endotoxin ratio 10 ng/0.90 ml = 100 EU/ml]; Cambrex, Verviers, Belgium) with the maximal reaction rate (V_{max}) as derived from kinetic readings with 30-s intervals (V_{max} in milli-optical density units [mOD]/min) as the primary outcome parameter for each test well. The endotoxin concentrations in the extracts were determined by comparing the V_{max} values in the test wells with the V_{max} calibration curve obtained with serial dilutions of the LPS standard in the same microplate.

In the first experiment, all extracts were analyzed in duplicate at various dilutions (1:2 to 1:400, depending on the type of dust and the medium used), and the assay, including dilution of the samples, the standard, and the LAL reagent, was performed in the same medium as was used for extraction of the filters. Samples with nondetectable endotoxin levels were assigned a value of two-thirds of the limit of detection (LOD) of the particular assay run (range, 0.01 to 0.06 EU/ml).

In the crossover experiment, the selected sample extracts were analyzed in three dilutions (1:90, 1:270, and 1:810). The mean of the results that were greater than the LOD was used in further statistical analyses. When all results were less than the LOD, a value of two-thirds of the LOD for that particular test, depending on the assay medium, was assigned.

Statistical analysis. Data were analyzed with SAS statistical software (version 9e; SAS Institute, Cary, NC). The endotoxin concentrations were log-normally distributed. Therefore, all calculations were performed with natural log-transformed concentrations. The influence of, differences between, and possible interactions between extraction and assay media were determined by applying mixed-effects analysis of variance with sampling run as the random effect, in order to correct for possible correlation between measurements in the same run. Determinants influencing the endotoxin concentration, i.e., the extraction and assay media, were explored by introducing them into the model as fixed effects (17, 19).

RESULTS

Of the 250 parallel airborne dust samples collected, endotoxin levels below the LOD were found in duplicate tests of 14 samples and for 5 samples, one of the two values was below the LOD. The endotoxin concentration of 14 out of 15 field blanks was below the LOD and in one was 0.8 EU/ml. Three of the

TABLE 1. Components of between- and within-run variance and effects of extraction medium on measured endotoxin levels (in EU/ml) in parallel airborne dust samples from five work environments relative to extraction (60 min) and analysis in PFW as the reference procedure[a]

Source (no.) of samples	Between-run variance	Within-run variance	Geometric mean with PFW, 60 min	PFW, 10 min		PFW-Tween, 60 min		TAP, 60 min		Tris, 60 min	
				e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI
All sources (250)	4.36	0.19	10.80	1.05	0.89–1.25	3.47*	2.92–4.12	0.67*	0.56–0.79	0.96	0.80–1.14
Household waste composting (50)	0.39	0.08	2.31	1.03	0.80–1.32	2.09*	1.63–2.69	0.98	0.76–1.26	1.30*	1.02–1.67
Grass seed production (50)	0.94	0.03	57.80	1.11	0.94–1.31	3.59*	3.04–4.24	1.64*	1.39–1.93	0.98	0.83–1.16
Pig stable (50)	0.55	0.06	55.87	0.99	0.80–1.24	2.89*	2.32–3.60	0.26*	0.20–0.32	0.83	0.67–1.03
Potato processing (50)	1.30	0.02	34.45	1.01	0.90–1.14	3.48*	3.09–3.92	0.82*	0.73–0.93	0.98	0.87–1.10
Sewage treatment (50)	0.78	0.23	0.57	1.12	0.72–1.73	6.62*	4.28–10.24	0.40*	0.26–0.61	0.77	0.50–1.19

[a] e^{β}, effect of the procedure relative to the reference procedure of PFW and 60 min of shaking; CI, confidence interval; *, $P < 0.05$.

samples from the crossover experiment, all extracted and analyzed in PFW, showed values below the LOD. The average coefficients of variation of duplicate analyses and intratest coefficients of variation of the results of serial dilutions of the same extract were 15% and 18%, respectively.

Comparison of different media used in both extraction and the LAL assay and of extraction time. Extraction in PFW with 10 min of shaking did not significantly change the endotoxin yield in comparison to the yield of extraction in PFW with 60 min of shaking, and this was the case for samples from all five work environments (Table 1). This confirms that there was no additional release of endotoxin during the longer period of shaking. The presence of Tris in the extraction medium and during analysis also had no significant effect on the overall measured endotoxin concentration, although the results suggested some heterogeneity in endotoxin release among workplaces, reflected by relative effects that were <1 except for household-waste composting (Table 1). The use of TAP instead of PFW showed on average a lower endotoxin yield ($P < 0.05$). The data suggested an even-more pronounced dependence on workplace, with decreased yields in samples from three workplaces (pig farm, potato processing, and sewage treatment), no significant effect in samples from one workplace (household-waste composting) and a significant and substantial increased yield in samples from the grass seed production. The addition of Tween 20 to PFW increased the endotoxin yield both overall and for the different work environments, with factors ranging from 2.1 to 6.6 (Table 1).

Effects of assay media on the reactivity of the standard in the LAL assay. As shown in Fig. 2, the application of the four different dilution media to the LAL assay resulted in marked differences in the shapes of the four calibration curves (based on three curves per medium) and their positions relative to the *x* axis. Compared to the performance of the assay in PFW, the use of Tris resulted in a very similar curve, while for TAP a change in the dose response was observed, with decreased reactivity at low and increased V_{max} values at high concentrations. Thus, the assay in TAP appeared to have a lower sensitivity, with an approximately 10-fold-increased LOD compared to the LOD of the assay in PFW. The addition of Tween to PFW showed a consistent effect on the reactivity of the standard, with a downward shift of approximately 35 to 50 V_{max}

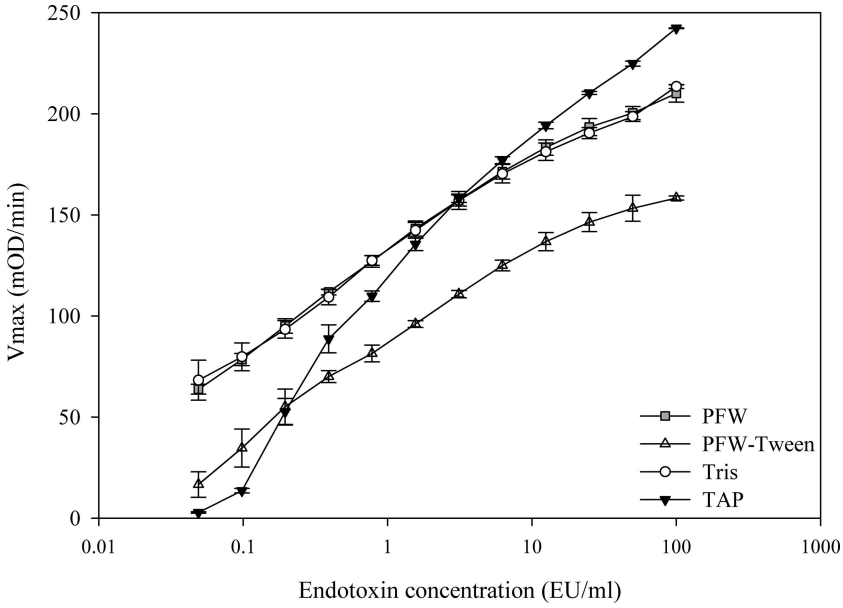

FIG. 2. Mean (and 95% confidence interval) standard curves of the results of the LAL assay performed using different dilution media, based on three standard curves per medium.

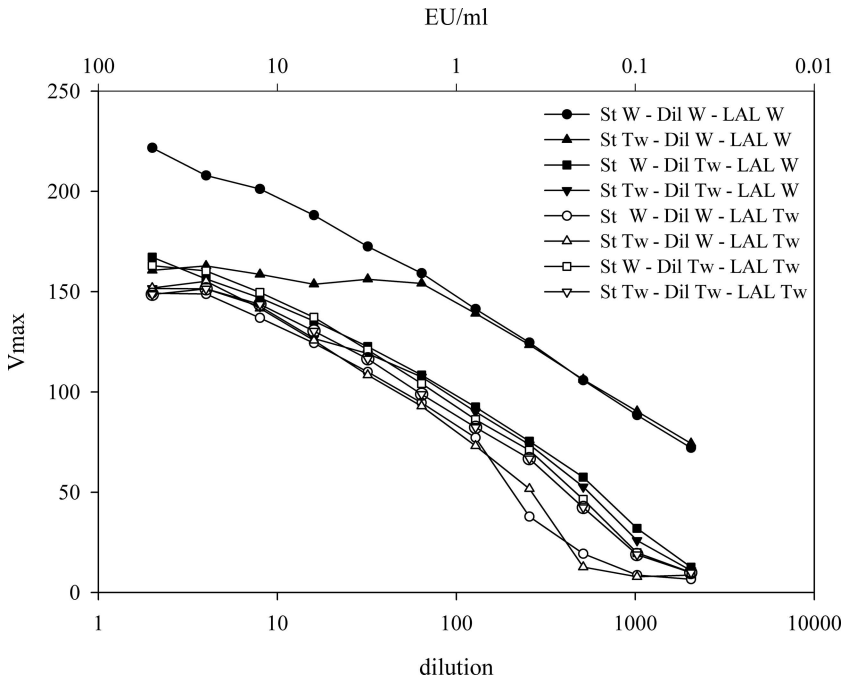

FIG. 3. Effect of Tween 20 on the reactivity of the LPS standard during analysis investigated in a full crossover experiment in which the standard was dissolved in PFW with or without 0.05% Tween 20, serially diluted in either PFW or PFW-Tween, and then tested with the LAL lysate reagent dissolved in either PFW or PFW-Tween. St, medium for dilution of LPS standard; Dil, diluting medium; LAL, LAL assay medium; W, PFW; Tw, PFW-Tween.

units (mOD/min) over the whole concentration range, resulting in a fivefold-increased LOD.

Effect of Tween on the reactivity of the calibration standard. As PFW-Tween showed the most-consistent results and because of previously reported effects (23), we further focused specifically on the effects of PFW-Tween in the extraction and/or assay medium. The shift of the standard curve was always observed when the standard was diluted in PFW-Tween and/or when the LAL reagent was prepared in PFW-Tween, irrespective of the Tween content of the primary standard solution (Fig. 3). The standard curve with the use of PFW-Tween for dissolving and further dilution of the standard and for preparation of the lysate was parallel to the calibration curve for the assay without Tween in each step over nine twofold dilutions but showed reduced reactivity. Preparation of the standard in PFW-Tween followed by dilution in PFW and preparation of the LAL reagent in PFW yielded a nonparallel curve that was kinetically invariant over the first six dilutions. At these first six dilutions, the V_{max} was markedly lower than in the complete absence of Tween. Though, from a dilution of approximately 50 to 100 and higher, V_{max} values were practically identical to those for the Tween-free tests (Fig. 3). Thus, the apparent inhibitory effect of Tween on the LAL assay was dose dependent and could be resolved if extracts containing 0.05% Tween 20 were diluted at least 1:50.

Testing of the LPS standard in PFW with various Tween concentrations confirmed the dose dependency of the inhibitory effect of Tween (Fig. 4). Tween concentrations of approximately 0.002% and higher caused a dose-dependent inhibition of reactivity in the LAL assay at all concentrations of the LPS standard.

Tween effect in airborne dust samples. To test whether Tween applied during extraction might result in an increased yield irrespective of the effect of Tween in the analysis phase, a crossover study was performed in a subset of the airborne dust samples. Figure 5A shows a high correlation ($r = 0.93$) between the results from analyses in PFW and PFW-Tween. Very similar values were found in both tests for samples from the pig stable and composting industry, because the inhibitory effect of Tween on their LPS reactivity in the assay was nearly identical to that of Tween on the LPS standard, thus with the V_{max} values in PFW-Tween approximately 40 to 45 mOD/min lower than in the absence of Tween. However, for samples from the other sources, the effect of Tween on the observed V_{max} values was markedly less, with a downward shift of only 25 to 30 mOD/min in the presence of Tween. As a result, the concentrations in samples from sewage treatment, potato industry, and grass seed-processing were significantly higher when the LAL assay was performed in PFW-Tween and sample results were calculated by comparison with the corresponding standard curves made in PFW-Tween. Irrespective of this test artifact, however, Fig. 5B ($r = 0.95$) shows a pronounced enhancing effect of Tween during extraction for all dust types, with a yield that was on average 5 to 10 times higher with the presence of Tween during extraction. Enhanced release of endotoxins from airborne dust filters was observed over a wide range of endotoxin levels, with some indications that this effect might be relatively stronger in samples with a lower endotoxin concentration.

Mixed regression analysis confirmed that extraction in PFW-Tween resulted in a significantly increased measured endotoxin concentration in the extracts of airborne dust samples

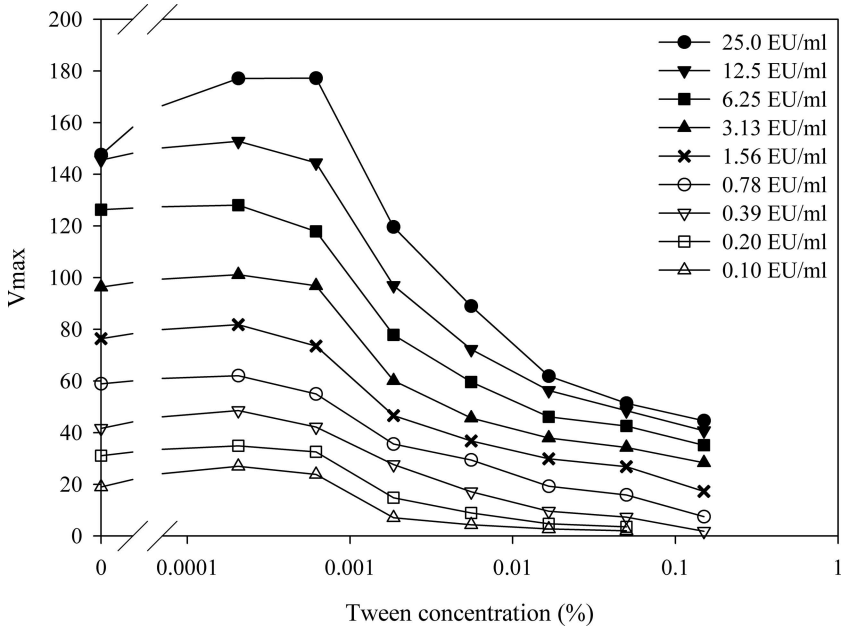

FIG. 4. Dilution series of LPS standard in PFW with various Tween concentrations.

from all work environments, both for assays in PFW (on average fivefold higher) and for assays in PFW-Tween (on average fourfold higher) (Table 2). The presence of Tween in the assay medium led to more-heterogeneous results. For all airborne dust samples except those taken during composting, the addition of Tween to the assay medium resulted in higher calculated endotoxin concentrations for samples extracted in PFW. For samples extracted in PFW-Tween, the enhancing effect of Tween in the assay medium on the calculated endotoxin concentrations was on average 1.8 and varied between 0.7 and 4.8 depending on the location of sampling (Table 2).

DISCUSSION

Previous studies have shown that the addition of Tween 20 to the extraction medium (PFW) results in higher measured endotoxin concentrations in the LAL assay (7, 23). This effect has usually been ascribed to enhanced extraction efficiency in the presence of Tween. However, we had preliminary results showing an additional effect of Tween in the actual LAL assay (unpublished data). The previously reported apparently higher yields in the presence of Tween could thus be partially due to a change in the reactivity of LPS in the LAL assay, especially if the reactivity of LPS in extracts of airborne dust samples and that of the calibration standard would be differentially affected by Tween in the assay medium. Therefore, this study looked at the effect of the medium during extraction and analysis separately.

The assay medium with Tween showed markedly lower V_{max} values for the standard LPS and therefore led to a decreased assay sensitivity. This inhibitory effect of Tween on the reactivity of LPS was similar for airborne dust samples from two work environments (pig farming and composting) but less for samples from three other sources (sewage treatment, grass seed, and potato processing). This resulted in higher measured endotoxin concentrations in the latter samples when analyzed in LAL assays in the presence of Tween, while the concentrations in the former were independent of the assay medium. Furthermore, this inhibitory effect of Tween seemed to be stronger when the samples had also been extracted in PFW-Tween.

An explanation for the Tween-related assay inhibition is not directly available. The effect of Tween in the assay medium appeared to be reversible, and interference of Tween from the extraction medium in the LAL assay can thus be avoided if extracts are sufficiently diluted. Tween might change the tertiary structure of LPS molecules or interfere with one or more of the (pro-)enzymes of the LAL reagent. As a surfactant, Tween might reduce the availability of LPS by partially capturing it in micelles, or it might affect the molecular interactions with and between the LAL factors. Since the effect differed between the (semi)purified LPS standard and LPS in the dust samples from various work environments, an effect on extracted or dissolved LPS is the most likely explanation. However, little is yet known about the precise physical-chemical properties and appearance of LPS in organic dust extracts (level of aggregation and linkage to other macromolecular complexes) and their impact on its in vivo and in vitro proinflammatory effects and LAL assay reactivity.

We found a higher endotoxin yield when samples were extracted in PFW-Tween than in PFW that was independent of the assay medium and showed that the effect of extraction and analysis in the presence of Tween is not only due to the change in assay reactivity but results from increased extraction efficiency. The results found in earlier studies (7, 23) thus were likely caused by an enhanced extraction efficiency. Disruption of hydrophobic interactions between LPS and filter material caused by the surface-active properties of Tween and disaggregation of endotoxin-containing molecular complexes from the cell wall of whole bacteria or from cell walls have been

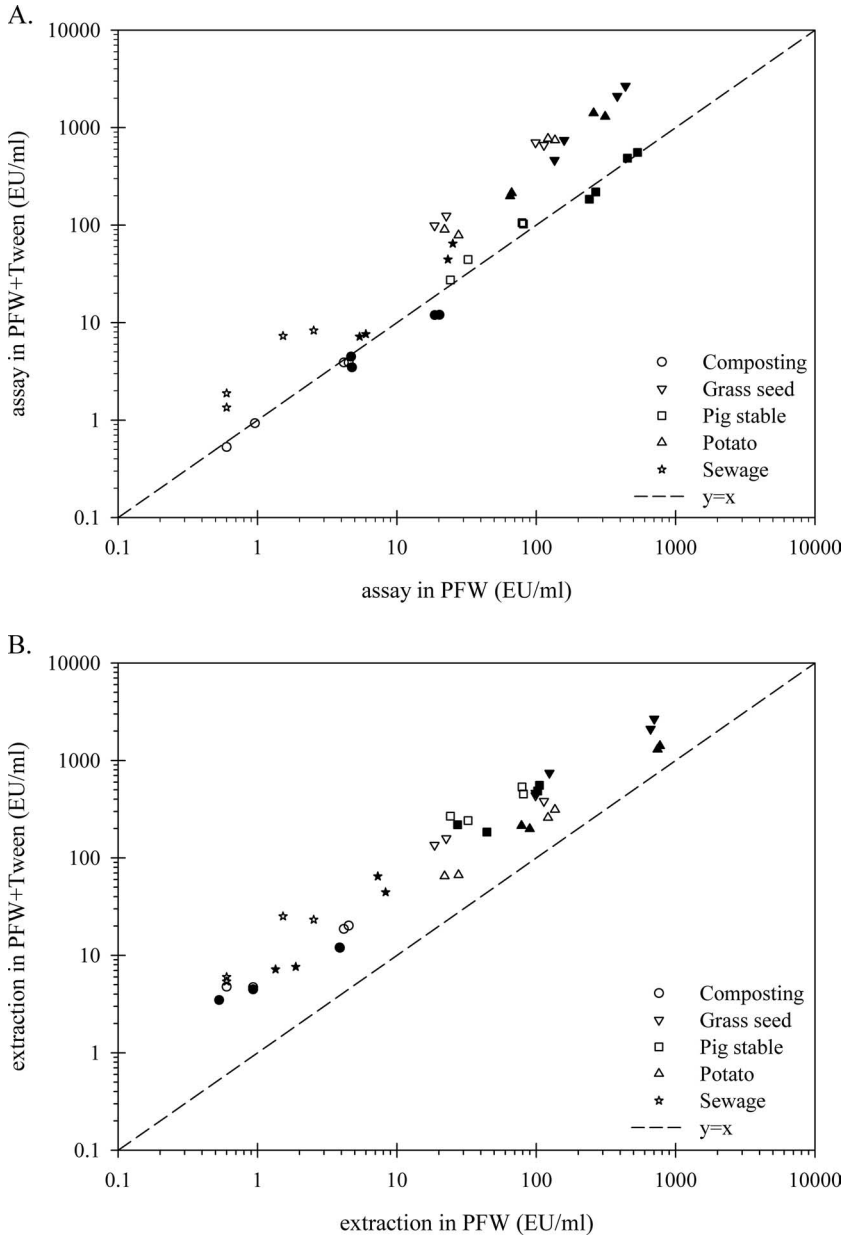

FIG. 5. Plots of the assay effect, stratified for extraction solution, of assays performed in PFW-Tween versus PFW on measured endotoxin concentration (EU/ml) for samples extracted in PFW (open symbols) or PFW-Tween (closed symbols) (A) and the extraction effect, stratified for assay solution, of extraction in PFW-Tween versus PFW on measured endotoxin concentration for extracts analyzed in PFW (open symbols) or PFW-Tween (closed symbols) (B). Key indicates type of production or processing facility sampled.

given as possible explanations (6, 7). Furthermore, Tween might reduce the sticking of LPS to the walls of tubes and vials used for extraction, storage, or dilution. However, the Tween effects in the earlier studies appeared to be independent of the use of different types of tubes and vials, which argues against this explanation (7).

The presence of Tween during extraction significantly increased the endotoxin yield for all dust types tested. In an analogous experiment with bulk dust samples, we found a similar but less-pronounced effect (mean relative effect of 2.9 for bulk dust versus 5.6 for airborne dust samples, data not shown). This suggests that the addition of Tween during the extraction procedure enhances the release of LPS from its natural matrices as well as from filters. However, the effect of the addition of Tween to the extraction and/or assay medium showed some heterogeneity with the type of dust or work environment, although the small number of samples this observation is based on precludes firm conclusions. Differences between sampled environments have been reported before (21, 23). The fact that the effect of Tween on the extraction efficiency seemed somewhat higher at lower endotoxin concentrations might partly explain the now-observed differences.

Other buffers and dispersing agents have also been proposed to increase extraction efficiency and/or to stabilize the pH and

TABLE 2. Effect of PFW-Tween (compared to PFW) in the extraction and assay media on endotoxin yield (in EU/ml), stratified for assay medium and extraction medium, respectively[a]

Source (no.) of samples	Extraction effect (PFW-Tween vs. PFW)				Assay effect (PFW-Tween vs. PFW)			
	Assay in PFW		Assay in PFW-Tween		Extraction in PFW		Extraction in PFW-Tween	
	e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI
All sources (40)	5.58*	4.50–6.92	4.08*	3.40–4.90	2.51*	1.86–3.38	1.83*	1.35–2.49
Household waste composting (8)	5.28*	3.77–7.39	4.16*	2.53–6.83	0.91	0.60–1.39	0.72*	0.57–0.90
Grass seed production (8)	5.24*	3.52–7.80	4.29*	2.84–6.49	5.89*	4.74–7.32	4.82*	3.27–7.13
Pig stable (8)	7.45*	5.51–10.07	5.36*	3.91–7.36	1.27	0.92–1.76	0.91	0.72–1.16
Potato processing (8)	2.43*	1.96–3.00	2.09*	1.70–2.58	4.47*	3.13–6.39	3.86*	2.94–5.07
Sewage treatment (8)	10.80*	7.72–15.11	5.66*	3.89–8.23	3.24*	2.12–4.96	1.70*	1.16–2.49

[a] e^{β}, effect of the procedure relative to the reference procedure; CI, confidence interval; *, $P < 0.05$.

ionic strength of the extract in the LAL assay (6). In this study, the use of Tris for extraction (and during analysis) resulted in endotoxin concentrations which were comparable to those found in PFW, and the use of TAP lowered the measured endotoxin concentration compared to that obtained with PFW, with relatively large variations depending on the kind of dust investigated. Furthermore, the use of TAP showed a deviant calibration curve, with a lower sensitivity. Our findings for Tris and TAP are consistent with findings reported earlier (9, 28). Based on the consistent results with Tween, we decided to only further investigate the separate effect of Tween during extraction and analysis, although similar experiments could have been done with TAP or Tris. For instance, Laitinen mentioned an average 17% decrease in endotoxin concentration when a Trizma buffer was present in the assay compared to the concentration with PFW and a 25% increase when a KH_2PO_4 buffer was used, but details were not reported (9).

Rocking, sonication, or a combination of both are the most-commonly used methods for extraction of filters in an extraction medium. Additionally, the temperature during the extraction may be altered. We found no differences in endotoxin yield after extraction in PFW with 10 or 60 min of shaking, which indicates that a longer extraction duration, at least after a certain time of vigorous rocking, does not result in increased endotoxin yields. Likewise, others found no difference between results with gentle and vigorous rocking for 1 h at either room temperature or 60°C (7) or in endotoxin activity from 120 min of vigorous shaking at 22°C or 30 min of gentle rocking at 68°C (28).

Tween in the extraction medium thus on average clearly increases the efficiency of extraction and the availability of LPS in the assay. However, the actual extraction efficiency of airborne endotoxins from filters after sampling is still unknown and remains to be investigated. In the case of allergen extraction, 20 to 25% of allergens could be additionally released and measured in extracts after a second extraction of filters (1). In the study of Laitinen, spiking standard endotoxin on several filter types revealed recovery rates of 70 to 100% from filters that were placed in PFW directly after the spiking and highly variable recovery rates of 5 to 90% from filters that were dried first, with the percentage of recovery depending on the filter type. The highest recovery rates were from glass fiber filters (9). Spiking of electrostatic wiping cloths with house dust of defined endotoxin content resulted in 37 to 96% recovery rates (27).

Differences between laboratories in measured endotoxin concentrations of parallel samples have been reported (20, 21), which poses a serious problem when an exposure limit has to be estimated or compliance with an exposure limit is required. It has been suggested that the harmonization of protocols can lead to more-comparable results (5, 10). Although the CEN-14031 protocol is meant to provide a protocol for the measurement and analysis of airborne-endotoxin concentrations (4), some parameters are left unspecified, and in practice, many different protocols are used. This is also the case for the American Society for Testing and Materials' method for the analysis of endotoxins in metal-working fluids (26).

Based on the results of this study and previously reported results (23), it is recommended that airborne-endotoxin samples should be extracted in PFW plus 0.05% Tween 20 to obtain optimal endotoxin yields. In the case of airborne (and bulk) dust samples in which dilution factors of at least 50 can be applied to fully rule out a possible effect of the presence of Tween in the extract on the LAL assay, we propose analysis of the extracts in PFW. The sensitivity of the LAL assay in PFW is higher, which allows for the determination of relatively low endotoxin concentrations (0.05 EU/ml) and thus also the application of relatively high dilution factors. The suggested dilution factor of 50 might even be too conservative, as in the application of airborne house dust samples, it was found that dilution factors of 25 led to essentially the same results as analyses with extracts diluted 1:50 (I. Noss, I. M. Wouters, M. Visser, D. J. J. Heederik, P. S. Thorne, B. Brunekreef, and G. Doekes, submitted for publication). Our results have also revealed a better reproducibility of the standard curve in PFW than in PFW-Tween, especially when switching over to a new batch of LAL assay reagent (data not shown). This might be due to practical difficulties when handling Tween-containing medium, which may cause the actual amount to vary during pipetting. The presence of Tween in the assay may also be a source of other inaccuracies, and we therefore prefer PFW as the assay medium. At this moment, however, there is too little information available to extrapolate these findings to samples with another origin or constitution or samples with a very low endotoxin content that does not allow much dilution to be detectable, like medical fluids, cell culture media, or pharma-

ceutical samples. Neither is it possible to introduce the relative effects for the different dust types observed in this study as conversion factors for a certain environment or kind of dust until they are shown to be reproducible. Finally, the physicochemical manner by which Tween enhances endotoxin extraction and the LAL assay should be further investigated, which would require studies on a molecular level.

ACKNOWLEDGMENTS

We acknowledge the following individuals for their contributions to this work: the company owners who cooperated and made this study possible, Marc Lurvink and Roel Engel (TNO) for conducting the fieldwork, and Hans Jongsma and Jack Spithoven (IRAS) for their analytical assistance.

This research was funded by the Ministry of Social Affairs and Employment, The Netherlands, through the long-term research program on biological agents in the work environment of TNO Quality of Life.

REFERENCES

1. **Bogdanovic, J., A. J. de Pater, G. Doekes, I. M. Wouters, and D. J. Heederik.** 2006. Application of porous foams for size-selective measurements of airborne wheat allergen. Ann. Occup. Hyg. **50:**131–136.
2. **Bogdanovic, J., I. M. Wouters, I. Sander, E. Zahradnik, H. R. Joanne, M. J. Rodrigo, S. Gomez-Olles, D. J. Heederik, and G. Doekes.** 2006. Airborne exposure to wheat allergens: optimised elution for airborne dust samples. J. Environ. Monit. **8:**1043–1048.
3. **CEN.** 1998. 13098. Workplace atmosphere: guidelines for measurement of airborne micro-organisms and endotoxin. European Committee for Standardization, Brussels, Belgium.
4. **CEN.** 2000. 14031. Workplace atmosphere: determination of airborne endotoxins. European Committee for Standardization, Brussels, Belgium.
5. **Chun, D. T., K. Bartlett, T. Gordon, R. R. Jacobs, B. M. Larsson, L. Larsson, D. M. Lewis, J. Liesivuori, O. Michel, D. K. Milton, R. Rylander, P. S. Thorne, E. M. White, M. E. Brown, V. S. Gunn, and H. Wurtz.** 2006. History and results of the two inter-laboratory round robin endotoxin assay studies on cotton dust. Am. J. Ind. Med. **49:**301–306.
6. **Cooper, J. F.** 1990. Resolving LAL test interferences. J. Parenter. Sci. Technol. **44:**13–15.
7. **Douwes, J., P. Versloot, A. Hollander, D. Heederik, and G. Doekes.** 1995. Influence of various dust sampling and extraction methods on the measurement of airborne endotoxin. Appl. Environ. Microbiol. **61:**1763–1769.
8. **Eduard, W., G. Blomquist, B. Herbert Nielsen, and K. Kulvik Heldal.** 2001. Recognition errors in the quantification of micro-organisms by fluorescence microscopy. Ann. Occup. Hyg. **45:**493–498.
9. **Laitinen, S. K.** 1999. Importance of sampling, extraction and preservation for the quantitation of biologically active endotoxin. Ann. Agric. Environ. Med. **6:**33–38.
10. **Linsel, G., C. Doering, S. Duggal, J. Hartung, A. Koch, A. Kottmair, M. Lohmeyer, R. Schierl, K. Weist, and B. A. Zucker.** 2002. Ergebnisse eines Ringversuches zur Messung luftgetragener Endotoxine. VDI-Berichte **1656:** 329–339.
11. **Liu, A. H.** 2002. Endotoxin exposure in allergy and asthma: reconciling a paradox. J. Allergy Clin. Immunol. **109:**379–392.
12. **Milton, D. K., H. A. Feldman, D. S. Neuberg, R. J. Bruckner, and I. A. Greaves.** 1992. Environmental endotoxin measurement: the kinetic Limulus assay with resistant-parallel-line estimation. Environ. Res. **57:**212–230.
13. **Milton, D. K., R. J. Gere, H. A. Feldman, and I. A. Greaves.** 1990. Endotoxin measurement: aerosol sampling and application of a new Limulus method. Am. Ind. Hyg. Assoc. J. **51:**331–337.
14. **Milton, D. K., D. K. Johnson, and J. H. Park.** 1997. Environmental endotoxin measurement: interference and sources of variation in the Limulus assay of house dust. Am. Ind. Hyg. Assoc. J. **58:**861–867.
15. **Olenchock, S. A., D. M. Lewis, and J. C. Mull.** 1989. Effects of different extraction protocols on endotoxin analyses of airborne grain dusts. Scand. J. Work. Environ. Health **15:**430–435.
16. **Park, J. H., B. Szponar, L. Larsson, D. R. Gold, and D. K. Milton.** 2004. Characterization of lipopolysaccharides present in settled house dust. Appl. Environ. Microbiol. **70:**262–267.
17. **Peretz, C., A. Goren, T. Smid, and H. Kromhout.** 2002. Application of mixed-effects models for exposure assessment. Ann. Occup. Hyg. **46:**69–77.
18. **Portengen, L., L. Preller, M. Tielen, G. Doekes, and D. Heederik.** 2005. Endotoxin exposure and atopic sensitization in adult pig farmers. J. Allergy Clin. Immunol. **115:**797–802.
19. **Rappaport, S. M., M. Weaver, D. Taylor, L. Kupper, and P. Susi.** 1999. Application of mixed models to assess exposures monitored by construction workers during hot processes. Ann. Occup. Hyg. **43:**457–469.
20. **Reynolds, S. J., D. K. Milton, D. Heederik, P. S. Thorne, K. J. Donham, E. A. Croteau, K. M. Kelly, J. Douwes, D. Lewis, M. Whitmer, I. Connaughton, S. Koch, P. Malmberg, B. M. Larsson, J. Deddens, A. Saraf, and L. Larsson.** 2005. Interlaboratory evaluation of endotoxin analyses in agricultural dusts: comparison of LAL assay and mass spectrometry. J. Environ. Monit. **7:**1371–1377.
21. **Reynolds, S. J., P. S. Thorne, K. J. Donham, E. A. Croteau, K. M. Kelly, D. Lewis, M. Whitmer, D. J. Heederik, J. Douwes, I. Connaughton, S. Koch, P. Malmberg, B. M. Larsson, and D. K. Milton.** 2002. Comparison of endotoxin assays using agricultural dusts. Am. Ind. Hyg. Assoc. J. **63:**430–438.
22. **Rylander, R., and R. R. Jacobs.** 1997. Endotoxins in the environment: a criteria document. Int. J. Occup. Environ. Health **3:**S1–S48.
23. **Spaan, S., D. J. Heederik, P. S. Thorne, and I. M. Wouters.** 2007. Optimization of airborne endotoxin exposure assessment: effects of filter type, transport conditions, extraction solutions, and storage of samples and extracts. Appl. Environ. Microbiol. **73:**6134–6143.
24. **ter Kuile, W. M.** 1984. Vergleichmessungen mit verschiedenen Geräten zur Bestimmung der Gesamtsaubkonzentration am Arbeitsplatz: Teil II. Staub. Reihn. Luft. **44:**211–216.
25. **Thorn, J., L. Beijer, and R. Rylander.** 1998. Airways inflammation and glucan exposure among household waste collectors. Am. J. Ind. Med. **33:** 463–470.
26. **Thorne, P. S., K. H. Bartlett, J. Phipps, and K. Kulhankova.** 2003. Evaluation of five extraction protocols for quantification of endotoxin in metalworking fluid aerosol. Ann. Occup. Hyg. **47:**31–36.
27. **Thorne, P. S., N. Metwali, E. Avol, and R. S. McConnell.** 2005. Surface sampling for endotoxin assessment using electrostatic wiping cloths. Ann. Occup. Hyg. **49:**401–406.
28. **Thorne, P. S., S. J. Reynolds, D. K. Milton, P. D. Bloebaum, X. Zhang, P. Whitten, and L. F. Burmeister.** 1997. Field evaluation of endotoxin air sampling assay methods. Am. Ind. Hyg. Assoc. J. **58:**792–799.
29. **von Mutius, E., C. Braun-Fahrlander, R. Schierl, J. Riedler, S. Ehlermann, S. Maisch, M. Waser, and D. Nowak.** 2000. Exposure to endotoxin or other bacterial components might protect against the development of atopy. Clin. Exp. Allergy **30:**1230–1234.
30. **Walters, M., D. Milton, L. Larsson, and T. Ford.** 1994. Airborne environmental endotoxin: a cross-validation of sampling and analysis techniques. Appl. Environ. Microbiol. **60:**996–1005.